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14. ABSTRACT

The PTEN tumor suppressor gene is localized to the 10q23 interval and biallelic inactivation of PTEN has been demonstrated in prostate cancer cell lines, and in human primary and metastatic prostate cancers. In mice, inactivation of PTEN leads to prostate hyperplasia, PIN, and microinvasive cancers. Thus, PTEN is a bona fide tumor suppressor with special relevance to prostate cancer. We previously demonstrate that loss of PTEN protein was strongly associated with high-grade Gleason tumors and Suzuki et. al. showed that 56% of metastatic prostate cancer samples were PTEN null. Thus, loss of PTEN appears to correlate with clinical parameters known to be associated with poor outcome. These data suggest that small molecule inhibitors that act to reverse the transformed phenotype induced by loss of PTEN may find significant efficacy in lethal forms of prostate cancer. Loss of PTEN function leads to activation of the PI3K pathway, phosphorylation and activation of Akt and the subsequent aberrant phosphorylation and hence constitutive localization of the FOXO transcription factors to the cytoplasm of PTEN null cells. Based on these findings, we developed a novel, high-throughput cell-based screen using FKHR localization in PTEN null cells as a read-out. This screen has been conducted and numerous new lead compounds were identified. This grant focused on the evaluation of these lead molecules, their activity in in vitro and in vivo models, as well as their possible cooperation with other therapeutics targeting the same pathway.

15. SUBJECT TERMS
PTEN, Prostate cancer, Forkhead, FOXO, phenothiazines, calmodulin, Phosphoinositide-3

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INTRODUCTION

The *PTEN/MMAC/TEP-1* tumor suppressor gene (hereafter referred to as *PTEN*) is a target of somatic mutation in prostate cancer as well as in endometrial cancer, glioblastoma and melanoma (reviewed in (Sansal and Sellers, 2004)). Biallelic loss of *PTEN* has been demonstrated in both primary and metastatic prostate tumors (reviewed in (Sansal and Sellers, 2004)). In metastatic disease, *PTEN* loss approaches 50%-60% (Suzuki et al., 1998). Together, these data suggest that loss of PTEN is an important step for those prostate tumors associated with a lethal outcome. Moreover, the loss of PTEN has been intimately linked to deregulation of the PI3K pathway connecting growth and survival signals both to the regulation of the mTOR kinase as well as to the regulation of the FOXO transcription factors. A significant effort is now being expended in the pharmaceutical industry in trying to develop regulators of the PI3K pathway (or more specifically inhibitors) that can reverse the molecular consequences of PTEN loss.

Our group was among the first to publish data that FOXO proteins are aberrantly localized to the cytoplasm upon loss of functional PTEN and that FKHR localization is restored upon reconstitution of PTEN to PTEN null cells (Nakamura, et. al. 2000). Moreover, we went on to show that reconstitution of FOXO activity to the nucleus was sufficient to replace the tumor suppressor function of PTEN (Nakamura, et. al. 2000). Surprisingly this activity was not linked to the transactivation activity of FOXO but was linked to the ability of FOXO to repress cyclin D1 (Ramaswamy et. al. 2002). In total these data suggest that to a first approximation, small molecules that recapitulate this activity of PTEN, i.e. lead to re-distribution of FKHR from the cytoplasm to the nucleus, should lead to inhibition of cell-cycle progression and suppression of tumorigenicity of PTEN null cells.

These data led us to ask whether a novel cell-based small-molecule screen could be developed using FKHR localization as an end-point. Preliminary data showed that this was feasible and led to the discovery of novel small molecule inhibitors of the PI3K pathway (Kau et. al. 2003). Based on these results we proposed 3 specific aims:

1) To determine the mechanism of action of inhibitors that specifically re-localize FKHR to the nucleus
2) To determine the in vitro biological activity of small molecule inhibitors discovered in the FKHR screen.
3) To determine the in vivo anti-tumor efficacy of lead compounds in animal models.

Body

The role of phenothiazines in suppressing the PI3K/PTEN pathway in cellular assays.

In the prior report we provided data to demonstrate that 1) thioridazine administration or treatment of PTEN null cells led to a decrease in the phosphorylation of AKT at the S473 and T308 sites and that this was accompanied by a decrease in the phosphorylation of downstream targets including S6, GSK3and FOXO. We also demonstrated that Thioridazine could block S6K activity even in the absence of activation of AKT. These data raised the possibility that phenothiazines were acting on more than one point in the pathway or acting through PDK1. For additional details please refer to the DOD report from 1/24/05. In keeping with these data phenothiazines were anti-proliferative in cell lines

Anti-proliferative activity of thioridazine

Subsequently we have asked whether, given the inhibitory activity against the PI3K pathway whether phenothiazines are preferentially active against cells lacking the tumor suppressor gene PTEN. As shown

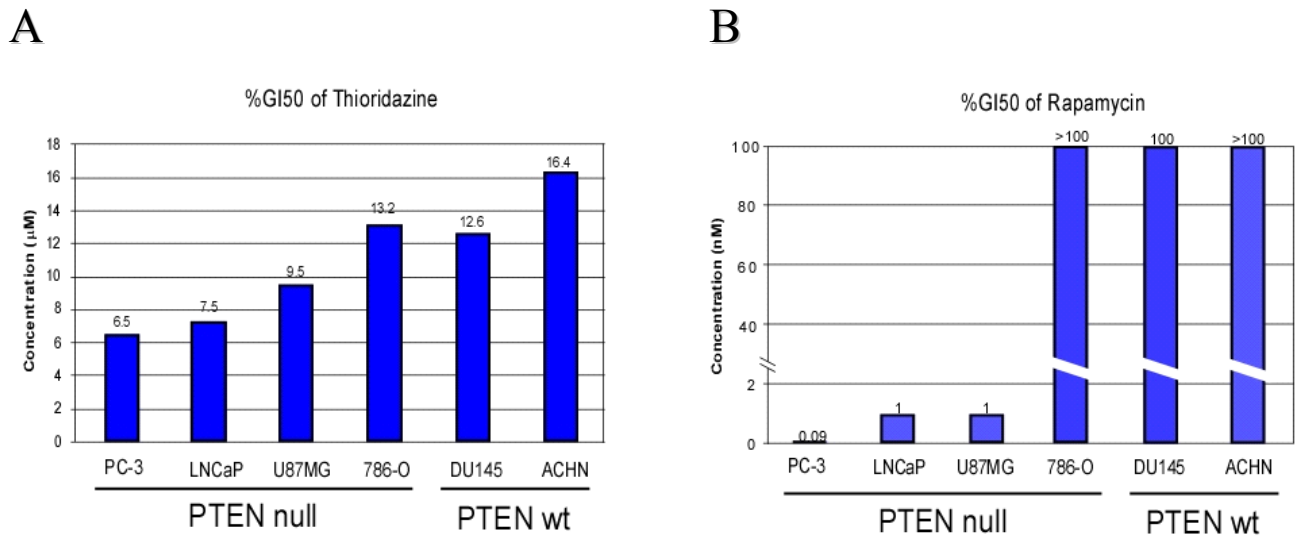

Figure 1: The indicated cell lines were treated with increasing concentrations of either thioridazine (A) rapamycn (B) and the concentration resulting in a 50% reduction in the rate of cell growth was determined and is shown on the Y-axis. In parallel control cells were treated with vehicle (DMSO).

in Figure 1A above – there is a trend towards this result. These results are concordant though not as dramatic as the results seen with rapamycin (Figure 1B). In both cases the PTEN null cells 786-O are outliers in this analysis for unknown reasons.

Is there synergy with rapamycin?

The data suggesting that thioridazine could act both upstream and downstream of AKT raised the possibility that thioridazine and/or phenothiazines in general might act to block a well-described feedback loop in the PI3K/mTOR pathway. This feedback loop dramatically unveiled in TSC null fibroblasts results in up regulation of AKT activity upon down regulation of mTOR activity. Thus mTOR inhibitors such as rapamycin while inhibiting S6K activity can lead to increased AKT activity. We asked whether or not thioridazine could block this and hence act synergistically with rapamycin. Representative of these results

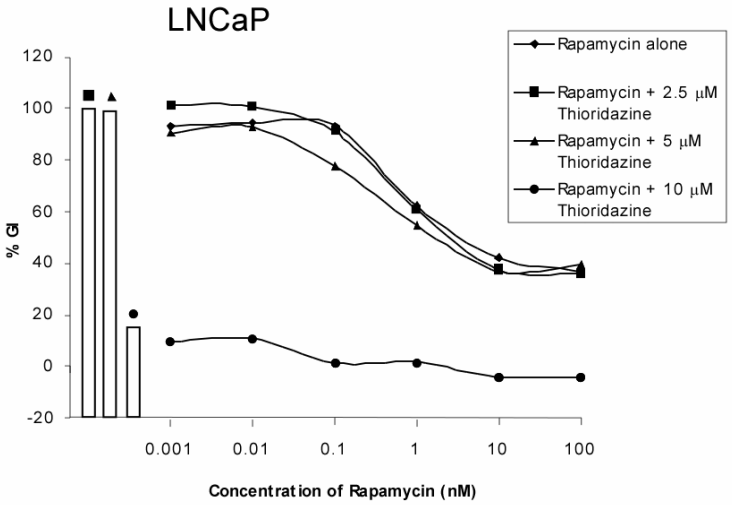

Figure 2: Thiordiazine does not synergize with rapamycin. PTEN null LNCaP cells were treated with thirodiazine alone at 2.5, 5 and 10 μm (bars) or the same concentrations of thioridazine with increasing concentrations of rapamycin. No synergy was demonstrated.

are those shown in Figure 2. Here we failed to demonstrate synergy between the phenothiazine thioridazine and rapamycin.

In vivo testing of phenothiazines in the Tg-AKT murine PIN model.

The focus for this past year was to determine whether phenothiazines were capable of inhibiting AKT signaling and inducing an appropriate phenotypic response in in vivo models. To this end we have used two different types of models. First, we have previously published work showing that a model in which activated human AKT1 is expressed in the prostate robustly develops PIN with very high penetrance (Majumder et. al. , 2003). Moreover, we have also published that in this model treatment with the rapamycin derivative RAD001 results in complete reversion of the PIN phenotype over at 15 day time course (Majumder et. al. 2004). This model thus allows us to test phenothiazines in a model that is known to be AKT dependent. To this end cohorts of mice were treated with either placebo or thioridazine given 10mg/kg for three doses. Mice were then sacrificed and the ventral lobe of the prostate was harvested and analyzed for AKT activity by staining with anti-phospho AKT antibodies. As shown in figure 3 – we observed no change in the level of AKT phosphorylation, nor any change in S6 phosphorylation (not shown). One likely possibility is that the dose of thioridazine administered to these animals was inadequate to achieve the concentration (or exposure) required to inhibit the pathway. While we do not have access to the tools required to assess the specific plasma AUC or tissue AUC of thioridazine we did

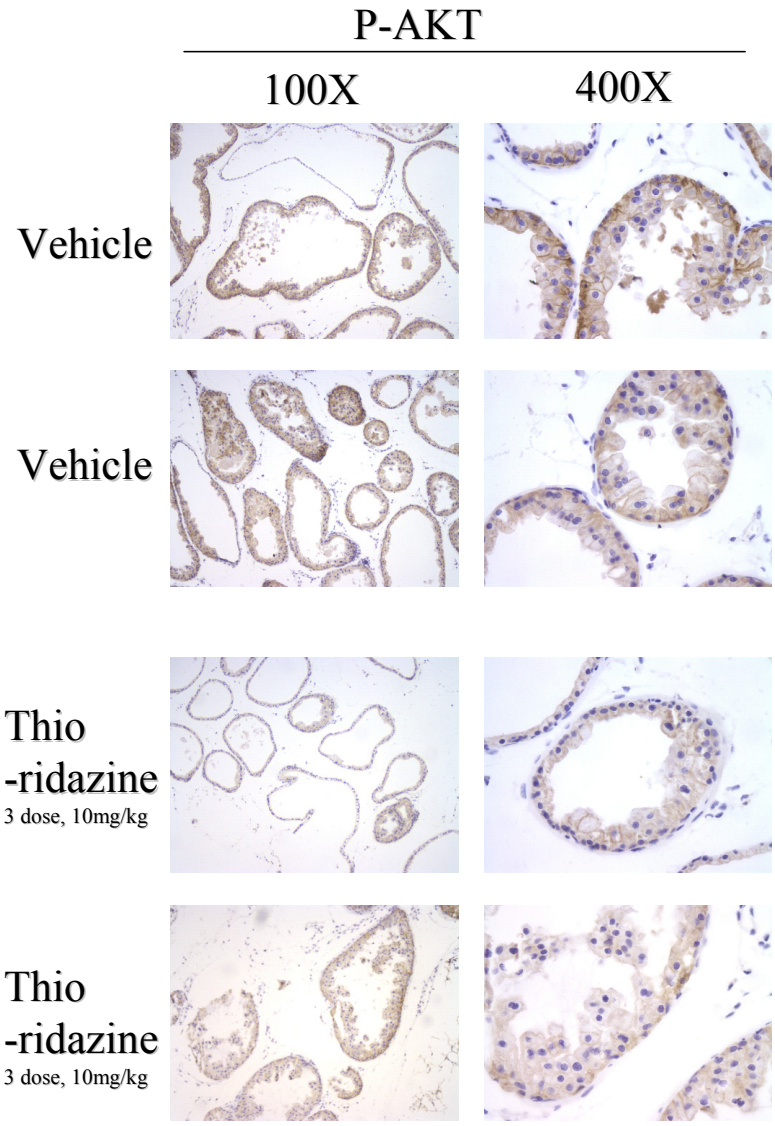

Figure 3: Thioridazine does not inhibit AKT phosphorylation in the Tg-AKT murine model. Cohorts of Tg-AKT mice were either treated with 3 doses of thoridazine or vehicle control as indicated. Sections prepared after harvesting the ventral prostate were stained with anti-Phospho-AKT antisera.

explore increasing doses of the compound. Unfortunately at doses higher than 10 mg/kg the mice were either lethargic or the dose was toxic and lethal to the animals. Indeed, it was difficult to administer more than three doses of the compound without encountering excess toxicity in the mice.

It is formally possible that such results might be obtained if the activation of Myr-HA-AKT1 was not subjected to regulation or inhibition by phenothiazines. To determine whether this was the case, RAT1A cells were transduced with a retrovirus directing the expression of Myr-HA-AKT1. Stable polyclonal pools of cells were derived and exhibited all of the hallmarks of transformation including growth in soft-agar and the induction of tumor growth in nude mice (data not shown). These cells were then treated in vitro with either vehicle alone or increasing concentrations of thioridazine. Here we were able to demonstrate robust inhibition of phosphorylation of Myr-HA-AKT1 and the inhibition of subsequent downstream signaling consequences as indicated by inhibition of phosphorylation of ribosomal protein S6 (Figure 4). From these data we conclude that thioridazine can inhibit PI3K signaling in vitro but there is no therapeutic index that allows inhibition of PI3K in murine models.

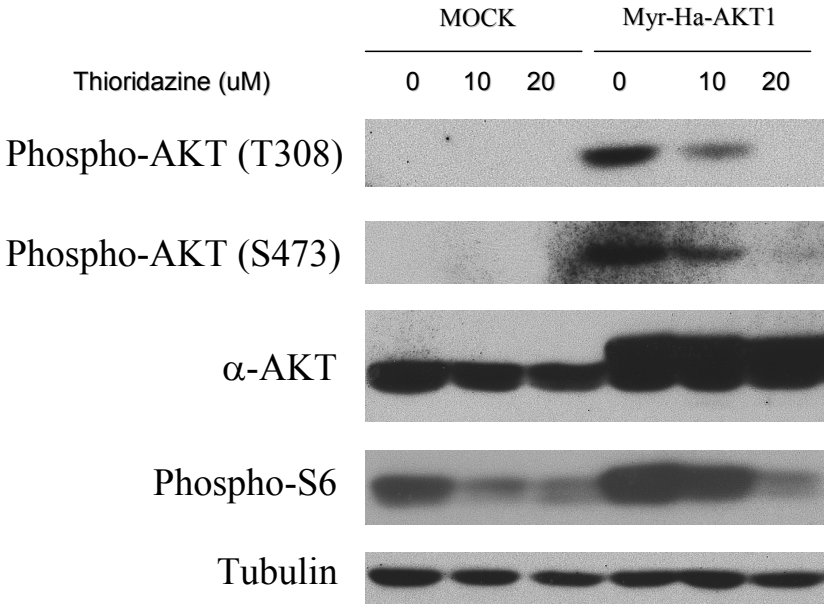

Figure 4: Thioridazine inhibits the phosphorylation of Myr-HA-AKT1 and the downstream phosphorylation of ribosomal protein S6. Myr-HA-AKT1 expressing RAT1A cells were treated with either vehicle or the indicated concentrations of Thioridazine for 6 hours. Cell lysates were prepared and separated by gel electrophoresis. The indicated protein species were detected by immunoblotting.

In vivo assessment of thioridazine in LNCaP xenografts.

The data above suggest that there may be limited ability to use phenothiazines as inhibitors of PI3K pathway inhibitors in vivo likely due to the relative low potency and also the dose-limiting toxicity (somnolence) linked to inhibition of dopamine receptor signaling. We tried one additional model where LNCaP xenografts were established in nude mice and again cohorts were treated with either vehicle alone or thioridazine administered as shown in figure 3. Again, we observed no inhibition of AKT phosphorylation (data not shown).

The identification of a natural product inhibitor of FOXO localization

Continued work resulting from the initial FOXO screen has resulted in the identification of a natural product inhibitor of the pathway. This work is described in the accompanying publication: Schroeder et al, The psammaplysenes, specific inhibitors of FOXO1a nuclear export. J Nat Prod. 2005 Apr;68(4):574-6.

KEY RESEARCH ACCOMPLISHMENTS:

Bulleted list of key research accomplishments emanating from this research.

- Demonstrated preferential growth inhibition of PTEN null cells for the phenothiazine class of drugs.
- Showed that phenothiazines can act both upstream and downstream of Akt in the PI3K pathway.
- Showed that phenothiazines can inhibit constitutively activated AKT (activated herein using a Myristoylation signal).
- Showed that phenothiazines are unable to inhibit the AKT pathway *in vivo* likely due to dose-limiting toxicity.

REPORTABLE OUTCOMES:

Provide a list of reportable outcomes that have resulted from this research to include:

1. Kau, T. R., Schroeder, F., Ramaswamy, S., Wojciechowski, C. L., Zhao, J. J., Roberts, T. M., Clardy, J., Sellers, W. R., and Silver, P. A.. A chemical genetic screen identifies inhibitors of regulated nuclear export of a Forkhead transcription factor in PTEN-deficient tumor cells. Cancer Cell 2003 *4*, 463-476.

2. Sansal, I., and Sellers, W. R. The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol 2004 *22*, 2954-2963.

3. Schroeder FC, Kau TR, Silver PA, Clardy J. The psammaplysenes, specific inhibitors of FOXO1a nuclear export. J Nat Prod. 2005 Apr;68(4):574-6.

4. Kau TR, Way JC, Silver PA. Nuclear transport and cancer: from mechanism to intervention. Nat Rev Cancer. 2004 Feb;4(2):106-17. Review.

5. Majumder PK and Sellers WR. Akt-regulated pathways in prostate cancer. Oncogene 2005. **24**(50): 7465-74.

CONCLUSIONS:

From these data we conclude that while phenothiazines are indeed antagonists of the PI3K pathway the doses required to achieve pathway inhibition can not be achieved in vivo likely due to dose-limiting toxicity linked to the anti-dopaminergic activity. These data argue that more potent calmodulin

inhibitors would be required to progress drugs acting this mechanism into in vivo settings. From these data we do not see utility in further in vivo testing of the phenothiazines in animal models.

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APPENDICES:

1. Schroeder FC, Kau TR, Silver PA, Clardy J. The psammaplysenes, specific inhibitors of FOXO1a nuclear export. J Nat Prod. 2005 Apr;68(4):574-6.

2. Majumder PK and Sellers WR. Akt-regulated pathways in prostate cancer. Oncogene 2005. **24**(50): 7465-74.

The Psammaplysenes, Specific Inhibitors of FOXO1a Nuclear Export

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A small collection of marine natural product extracts was screened for compounds that would compensate lost tumor suppressor functionality in PTEN-deficient cells. From the most active extract, the previously unreported bromotyrosine derivative, psammaplysene A (**1**), was identified. Psammaplysene A compensates for PTEN loss by relocalizing the transcription factor FOXO1a to the nucleus.

Cancer cells have gain-of-function or loss-of-function mutations, or both, that lead to unchecked cell proliferation. Small molecules can modulate gain-of-function mutations by inhibiting the mutated gene product,[1] but small molecule modulation of loss-of-function mutations has been quite difficult. Finding targets downstream of the loss-of-function mutation, which are amenable to small molecule modulation, is likely to be a more productive, if still unproven, approach. This paper describes the discovery of two previously unreported natural products from the marine sponge *Psammaplysilla* sp. These compounds were identified from a high-content screen for small molecules that restore the function of FOXO1a, a downstream target of the PTEN tumor suppressor.[2]

The path linking PTEN with FOXO1a involves several steps, not all of which are well understood (Figure 1). Loss of PTEN phosphatase activity has been noted in Cowden's disease, a hereditary disease with a marked predisposition for breast and thyroid cancers, and PTEN phosphatase deficiencies have been observed in many other cancers.[3] FOXO1a, a member of the Forkhead family of transcription factors, which negatively regulates cell cycle progression and cell survival, is an attractive downstream target for small molecule modulation of loss of PTEN function. As a result of loss of PTEN phosphatase activity, phosphorylated FOXO1a remains inappropriately localized in the cytoplasm and unable to restrain cell cycle progression.[4] Small molecules that would enforce the nuclear re-localization of FOXO1a would be, at a minimum, useful tools to investigate FOXO1a regulation and cell growth.

A cell-based screen to identify such small molecules used the subcellular localization of FOXO1a as a readout, and libraries of ~18 000 synthetic molecules (NCI Structural Diversity Set, Chembridge DiverSetE) and 352 uncharacterized extracts from the NCI collection were assayed in this primary screen.[2] Among the strongest screening positives was a dichloromethane/methanol extract from a marine sponge, *Psammaplysilla* sp., which was collected in the Indian Ocean.

For isolation of the active component, 200 mg of this extract was subjected to activity-guided fractionation. A simplified Kupchan solvent-partitioning scheme yielded hexane, dichloromethane, and methanol/water fractions, of which the dichloromethane fraction was most active. Further fractionation via silica gel chromatography followed by reversed-phase HPLC yielded two pure active compounds, which were named psammaplysene A (**1**, 7.2 mg) and psammaplysene B (**2**, 0.7 mg). Positive-ion electrospray ionization MS indicated formula weights of 765 and 751, respectively, whereby the isotopic pattern suggested the presence of four bromine atoms in both compounds. High-resolution electrospray MS gave $C_{27}H_{35}Br_4N_3O_3$ as the molecular formula for psammaplysene A. Further structural characterization using a standard set of 2D NMR experiments including dqf-COSY, NOESY, HMQC, and HMBC was straightforward. Psammaplysene A (**1**) and psammaplysene B (**2**) were characterized as previously unreported dimeric bromotyrosine alkaloids (Chart 1), each consisting of two modified dibromotyrosine units combined with fragments most likely derived from aliphatic amino acids.

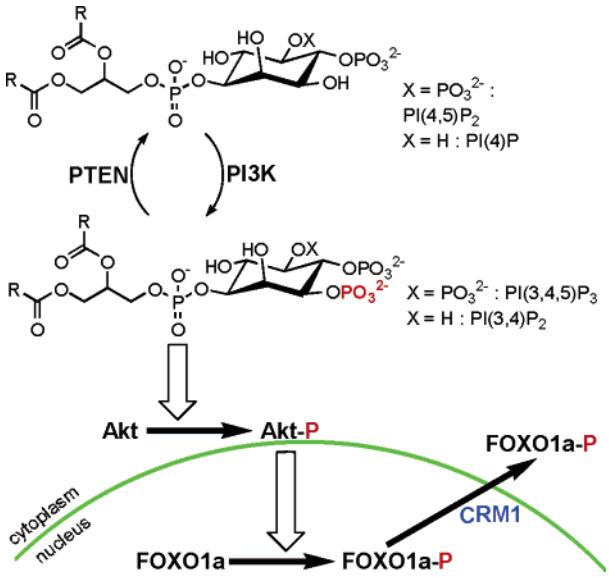

Figure 1. PTEN/PI3K/Akt/FOXO1a signaling pathway.[2] Akt phosphorylates FOXO1a, inducing FOXO1a nuclear export via CRM1. PTEN counteracts the kinase PI3K by dephosphorylating the lipid phosphates $PI(3,4)P_2$ and $PI(3,4,5)P_3$, thereby down-regulating phosphorylation of Akt and thus FOXO1a. In PTEN-null cells, FOXO1a remains constitutively phosphorylated and localized in the cytoplasm, which leads to cellular proliferation.

Psammaplysene A was among the most active inhibitors in this assay ($IC_{50} = 5\ \mu M$), whereas psammaplysene B was somewhat less active ($IC_{50} = 20\ \mu M$) (Figure 2). Among all of the compounds screened (>18 000), only five have IC_{50} values $\leq 5\ \mu M$.[2] Interestingly, the known dimeric bromotyrosine purpuramine-1 (**3**),[5] which was among the minor

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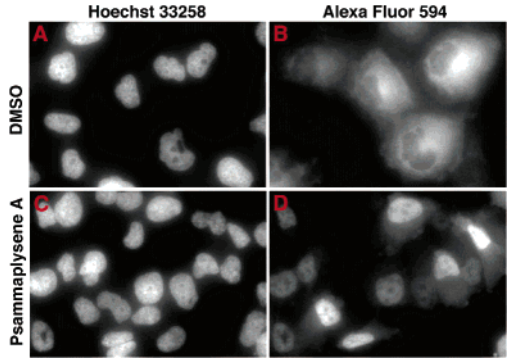

Figure 2. FOXO1a nuclear export inhibition by psammaplysene A (**1**). (A) PTEN-deficient cells[2] treated with DMSO (control) and stained with Hoechst 33258 to visualize nuclei and (B) stained with Alexa Fluor 594 to visualize localization of FOXO1a. Here, FOXO1a is predominantly in the cytoplasm. (C) cells treated with 5 μM psammaplysene A in DMSO stained with Hoechst 33258 and (D) stained with Alexa Fluor 594, showing re-localization of FOXO1a to the nuclei.

Chart 1. Structures of Psammaplysenes A (**1**) and B (**2**), Purpuramine-1 (**3**), Bastadin-5 (**4**), and Psammaplin A (**5**)

components in the extract, was not active in our assay. Purpuramine-1 and several homologous compounds, originally isolated from *Psammaplysilla purpurea*, were shown to have antibacterial properties.[6]

Sponges of the order Verongida to which the genus *Psammaplysilla* belongs are known to produce a wide range of structurally diverse bromotyrosine derivatives,[7] most prominent among them the sulfide-bridged psammaplins, such as psammaplin A (**5**), a histone deacetylase inhibitor,[8] and the macrocyclic bastadins, for example bastadin-5 (**4**),[9] a potent agonist of the RyR1 calcium channel.[10] The psammaplysenes (**1**, **2**) differ from related structures such as purpuramine-1 (**3**) and bastadin-5 (**4**) by having an α,β-unsaturated amide linkage and are distinguished by the way the two bromotyrosine subunits are connected.

There are multiple mechanisms to keep FOXO1a in the nucleus, and a series of secondary assays was used to assign the screening positives (42 including psammaplysene A) to distinct mechanistic classes. CRM1 is a general nuclear export receptor whose inhibition would localize FOXO1a and many other proteins in the nucleus.[11] A screen to assess whether compounds targeted CRM1 identified roughly half of the screening positives, 19 compounds, as CRM1 inhibitors and thus not specific inhibitors of the PI3K/Akt/FOXO1a signaling pathway. Psammaplysene A was not active in this secondary assay at 5 μM and thus considered pathway specific.[2]

Compounds specific for the PI3K/Akt/FOXO1a pathway could have targets upstream or downstream of Akt (Figure 1). By measuring levels of phosphorylated Akt by immunoblotting, the 23 pathway-specific compounds were assigned to these two classes. A total of 21 compounds led to decreased Akt phosphorylation, and two, including psammaplysene A, led to no change in Akt phosphorylation compared to untreated controls.[2] Psammaplysene A must have a target, which is as yet unidentified, downstream of Akt.

Among the five most potent positives in our primary screen, psammaplysene A (**1**) is the only compound that neither inhibited CRM1 nor reduced Akt phosphorylation. The target of psammaplysene A is not known, but small alterations of the basic structure, the removal of a methyl group to give psammaplysene B (**2**) for example, significantly diminish activity. The discovery of psammaplysene A's activity in this set of assays demonstrates that crude natural product extracts can be used to find potent and specific inhibitors in high-content, cell-based assays such as the one described. The highly modular psammaplysenes contain several easily accessible subunits and should therefore be amenable to synthesis-based exploration of structure−activity relations.

Experimental Section

General Experimental Procedures. NMR spectra were recorded at 25 °C using Varian INOVA500 (500 MHz proton, 126 MHz carbon) and Varian INOVA600 (600 MHz proton, 151 MHz carbon) spectrometers with CD_3OD or CD_2Cl_2 as the solvent. Double quantum filtered COSY (DQF-COSY) spectra were acquired using the standard pulse sequences and phase cycling. Phase-sensitive NOESY spectra were acquired with a mixing time of 600 ms. HMQC spectra were acquired in the phase-sensitive mode without gradients using phase-cycling for coherence selection. In some cases additional gradient HMQC and HSQC spectra were acquired. Magnitude-mode HMBC spectra were acquired without gradients and using phase-cycling for coherence selection. HMQC and HMBC spectra for psammaplysene B were acquired using Shigemi NMR tubes. Mass spectra were acquired using a Micromass Quattro II (positive-ion electrospray ionization), while high-resolution MS were obtained on a Micromass Autospec (positive-ion electrospray ionization). HPLC employed an Agilent 1100 series HPLC system with diode-array detector (190−900 nm) using a Supleco Discovery HS C-18 column (25 cm × 10 mm, 5 μm particle diameter).

Isolation of Psammaplysene A (1) and Psammaplysene B (2) by Activity-Guided Fractionation. Marine extract NCI-C013823-F3 (200 mg) was dissolved in 10 mL of a 9:1 mixture of methanol and water. The solution was extracted with two 10 mL portions of hexanes. Subsequently, the water content of the methanol phase was adjusted to 33%, followed by extraction with two 5 mL portions of dichloromethane. The resulting hexanes, dichloromethane, and methanol/water fractions were concentrated in vacuo and tested in

Table 1. ^{1}H and ^{13}C NMR Data of Psammaplysene A (**1**) (solvent CD_3OD, spectra referenced to 3.31 ppm for CD_2HOD, and 49.05 ppm for CD_3OD, coupling constants in Hz)

position	δ C	δ H	relevant HMBC correlations
1	56.6	3.48 (t, $J = 7$)	C-26/27
2	26.2	2.32 (quintet, $J = 7$)	
3	71.2	4.16 (t, $J = 7$)	C-4
4	154.24		
5	119.26		
6	132.8	7.82	C-4, C-10
7	135.8		
8	132.8	7.82	C-4, C-10
9	119.3		
10	137.6	7.39 ($J = 15.7$ Hz)	C-6/8, C-7, C-12
11	124.1	6.64 ($J = 15.7$ Hz)	C-12
12	167.5		
N−H		6.6 (br. t)	
13	36.6	3.60 (t, $J = 7$)	C-12
14	30.5	2.13 (quintet, $J = 7$)	
15	71.7	4.07 (t, $J = 7$)	C-16
16	153.2		
17	119.1		
18	134.1	7.59	C-16, C-19
19	136.8		
20	134.1	7.59	C-16, C-19
21	119.1		
22	30.3	3.00 (t, $J = 7$)	C-18/20, C-19
23	59.0	3.27 (t, $J = 7$)	C-19, C-24/25
24, 25	43.3	2.87 (s)	C-23, C-24/25
26, 27	43.4	2.94 (s)	C-1, C-26/27

our assay, which showed the dichloromethane fraction to be most active. For further purification, the dichloromethane fraction was chromatographed over silica using dichloromethane/methanol mixtures containing 0.4% concentrated aqueous ammonia as the solvent. Starting with 10% methanol, the methanol content of the solvent was gradually increase to 40%. Fractions eluting between 20 and 30% methanol were active and thus combined, concentrated, and subsequently rechromatographed using the same solvent system. This separation yielded two distinct fractions containing bromotyrosine-derived alkaloids. The earlier-eluting fraction contained almost pure purpuramine-1 (**3**), while the second fraction contained a mixture of psammplysenes A and B. The mixture of the two psammaplysenes was separated by reversed-phase HPLC using methanol/water mixtures with methanol contents of 35−100% as solvent, which yielded 7.2 mg of psammaplysene A (**1**, purity by NMR > 98%) and 700 μg of psammaplysene B (**2**, purity by NMR > 90%).

Psammaplysene A (1): ^{1}H and ^{13}C NMR data, see Table 1; positive-ion ESIMS *m/z* 775 (4), 774 (15), 773 (15), 772 (60), 771 (25), 770 (87), 769 (16), 768 (56), 767 (5), 766 (14) (ion cluster corresponding to M + H^+), 387.5 (20), 386.5 (68), 385.5 (100), 384.5 (70), 383.5 (18) (ion cluster corresponding to M + $2H^+$); positive-ion HRESIMS *m/z* 767.9443 (calcd for $C_{27}H_{36}{}^{79}Br_3{}^{80}BrN_3O_3$ 767.9470).

Psammaplysene B (2): ^{1}H and ^{13}C NMR data, see Table 2; positive-ion ESIMS *m/z* 761 (3), 760 (13), 759 (14), 758 (56), 757 (20), 756 (80), 765 (14), 754 (53), 753 (5), 752 (12) (ion cluster corresponding to M + H^+), 380.5 (19), 379.5 (69), 378.5 (100), 377.5 (69), 376.5 (17) (ion cluster corresponding to M + $2H^+$); positive-ion HRESIMS *m/z* 753.9247 (calcd for $C_{26}H_{34}{}^{79}Br_3{}^{80}BrN_3O_3$ 753.9314).

Table 2. ^{1}H and ^{13}C NMR Data of Psammaplysene B (**2**) (solvent CD_3OD, spectra referenced to 3.31 ppm for CD_2HOD, and 49.05 ppm for CD_3OD, coupling constants in Hz)

position	δ C	δ H	relevant HMBC correlations
1	49.0	2.83 (t, $J = 7$)	C-26
2	30.2	2.02 (quintet, $J = 7$)	
3	72.5	4.07 (t, $J = 7$)	C-4
4	154.1		
5	118.8		
6	131.9	7.66	C-4, C-10
7	133.9		
8	131.8	7.66	C-4, C-10
9	118.8		
10	137.0	7.40 ($J = 16$ Hz)	C-6/8, C-12
11	123.2	6.36 ($J = 16$ Hz)	C-12
12	164.8		
N-H		6.31 (br t)	
13	38.0	3.67 (t, $J = 7$)	C-12
14	29.8	2.10 (quintet, $J = 7$)	
15	72.3	4.10 (t, $J = 7$)	C-16
16	151.1		
17	117.9		
18	133.1	7.39	C-16, C-19
19	140.31		
20	133.1	7.39	C-16, C-19
21	117.9		
22	33.0	2.68 (t, $J = 7$)	C-18/20
23	60.8	2.47 (t, $J = 7$)	C-19, C-24/25
24, 25	45.2	2.21 (s)	C-23, C-24/25
26	36.2	2.43 (s)	C-1

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Supporting Information Available: ^{1}H NMR, DQF-COSY, coupled gHSQC, and HMBC spectra of psammaplysene A, as well as ^{1}H NMR, DQF-COSY, NOESY, HMQC, and HMBC spectra of psammaplysene B.

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Akt-regulated pathways in prostate cancer

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Prostate cancer remains a major cause of cancer-related mortality. Genetic clues to the molecular pathways driving the most aggressive forms of prostate cancer have been limited. Genetic inactivation of PTEN through either gene deletion or point mutation is reasonably common in metastatic prostate cancer and the resulting activation of phosphoinostide 3-kinase, AKT and mTOR provides a major therapeutic opportunity in this disease as mTOR inhibitors, HSP90 inhibitors and PI3K inhibitors begin to enter clinical development.
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Introduction

Prostate cancer is the second leading cause of cancer deaths in men. It is not invariably lethal, however, and is a heterogeneous disease ranging from asymptomatic to a rapidly fatal systemic malignancy. In 2005, the American Cancer Society estimates that there will be 234 300 new cases of prostate cancer and that 29 528 men will die of this disease (Jemal *et al*., 2005). Prostate cancers are typically detected through screening based on measurement of the serum prostate specific antigen (PSA) followed by prostate biopsy. The treatment of cancers confined to the prostate gland typically involves radical prostatectomy, external beam radiotherapy or radiotherapy delivered by seed implants (brachytherapy). While many patients with localized disease require no additional treatment, a subgroup will relapse and develop distant metastatic disease. Relapsed patients or patients who present with metastatic disease are treated by withdrawal of androgenic hormones either through medical castration using GNRH agonists or by orchiectomy. While the majority of patients will respond to hormone ablation, responses eventually give way to progressive, hormone-refractory prostate cancer. Additional therapeutic interventions including chemotherapy have some benefit, but of limited duration.

Prostate cancer can be divided epidemiologically into rare hereditary and the vastly more common sporadic forms. Although candidate inherited prostate cancer susceptibility genes have been identified such as the ELAC2, RNASEL, MSR1, NSB1 and CHEK2 genes, the proportion of cases of hereditary prostate cancer attributable to germline mutations in these loci is small and only occasional mutations in these candidate genes have been identified in sporadic prostate cancer (Hsieh *et al*., 2001; Xu *et al*., 2001; Casey *et al*., 2002; Rennert *et al*., 2002; Rokman *et al*., 2002; Xu *et al*., 2002).

The extent of somatic genetic alterations in prostate cancer is not fully understood. Primary prostate tumors are surrounded by stroma and metastatic tumors are typically localized to the bone. These factors contribute to the difficulty in obtaining high-quality tumor-enriched DNA suitable for genetic analysis. Negative mutations studies thus must be interpreted with great caution. Among the best characterized somatic genetic events are amplification of c-MYC and the androgen receptor (AR), mutation of p53, hemizygous deletion at 8p21 thought to target NKX3.1 and loss or mutation of RB1 (reviewed in Sellers and Sawyers, 2002). In 1997, the tumor suppressor gene *PTEN* was cloned from the 10q23 region, a region frequently targeted by loss of heterozygosity (LOH) in advanced cancers (Li and Sun, 1997; Li *et al*., 1997; Steck *et al*., 1997). Inheritance of a mutated germ line allele of PTEN is linked to the development of the related hamartoma syndromes Cowden disease (CD) and Bannayana–Zonana syndrome (BZS) (Liaw *et al*., 1997; Lynch *et al*., 1997; Nelen *et al*., 1997; Marsh *et al*., 1997a). CD is associated with an increased incidence of breast and thyroid malignancies (Marsh *et al*., 1997b); thus, germline mutations or PTEN confer an increased risk of malignancy. As will be detailed further below, somatic inactivation of PTEN is common in a number of cancers including prostate cancer, and over the past 8 years it has become clear that the loss of PTEN and subsequent activation of Akt is a critical event in human prostate cancer, and presents a pathway for rationally targeted molecular therapeutics.

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PTEN – a regulator of the phosphoinositide-3 kinase pathway

PTEN – a PIP3 phosphatase

The *PTEN* gene encodes a dual-specificity phosphatase active against protein substrates (Myers *et al*., 1997). Surprisingly, however, it has much better phosphatase activity against lipid substrates and in particular against the D3 phosphorylated position of phosphoinositide-3,4,5 trisphosphate (PIP3) (Maehama and Dixon, 1998). This lipid is the direct product of the phosphoinositide-3 kinase holoenzyme, suggesting that PTEN might act as a direct antagonist to the PI3K signaling pathway – a known critical oncogenic signaling pathway. Indeed, cells lacking an intact copy of PTEN harbor elevated levels of PIP3.

PI3K is a critical mediator of multiple signaling pathways. Simplistically, receptor tyrosine kinase growth factor receptors become activated and bind to the p85 regulatory subunit. This subunit binds to the catalytic subunit (p110) and activates PI3K. PI3K then phosphorylates the inositol ring of PI4P or PI4,5P2 at the D position to generate PI3,4P2 and PI3,4,5P3, which act as secondary messengers (Cantley and Neel, 1999).

From PIP3 to PI3K and AKT

Important downstream targets of PI3K and of PIP3 include the serine–threonine Akt kinase family (also known as PKB). PIP3, once generated in the plasma membrane, recruits Akt and PDK1 to the plasma membrane through an interaction between the phosphoinositide and the Akt or PDK1 pleckstrin homology domains (PH). Once recruited to the plasma membrane, Akt is phosphorylated and activated by PDK1 (Downward, 1998). Thus, PTEN null cells also harbor constitutively activated levels of Akt. For example, the prostate cancer cell lines PC3 and LNCaP harbor deletions and point mutation of PTEN, rendering each PTEN null. In these cells, basal levels of phosphorylated and hence active Akt exceed the levels of Akt seen in PTEN wild-type cells under conditions of serum stimulation.

Akt promotes both cell growth and cell survival by inactivating its downstream substrates including GSK3, BAD, FOXO and TSC2. Importantly, studies in *Caenorhabditis elegans* and *Drosophilla melanogaster* have linked activation of Akt to regulation of certain FOXO transcription factors and to the activation of mTOR and $p70^{S6K}$. Thus, as one might predict in human cancer cells lacking PTEN substrates of Akt including GSK3, FOXO proteins and TSC2 are also constitutively phosphorylated.

Linking tumor suppression by PTEN to regulation of PI3K signaling

While PTEN has been implicated in regulating non-PI3K pathway functions such as p53, the accumulating evidence supports the notion that transformation resulting from the loss of PTEN is likely mediated through dysregulation of the PI3K pathway. For example, the germline mutant PTEN; G129E retains the ability to dephosphorylate lipid substrates, but selectively lacks the lipid phosphatase activity (Myers *et al*., 1998; Ramaswamy *et al*., 1999). This germline mutation is associated with the identical phenotype as seen with mutations that render PTEN null for both lipid and protein phosphatase activity. In keeping with the central role of PI3K signaling downstream of PTEN inactivation, prostate cancer cells and other cancer cell lines lacking PTEN remain dependent upon activation of the PI3K pathway for growth and survival. Reconstitution of PTEN to such cells either arrests cells in G1 or induces apoptosis. PTEN reconstitution also suppresses the growth of PTEN-null prostate cancer cell lines in soft-agar and in nude mice. These phenotypic reversions also require the PTEN lipid phosphatase activity (Myers *et al*., 1998; Ramaswamy *et al*., 1999). Moreover, antagonizing signaling through the PI3K pathway can also revert the transformed phenotype of PTEN null prostate cancer cells. In a variety of mammalian systems, inactivation of Akt alleles, restoration of Forkhead activity or inhibition of mTOR and $p70^{S6K}$ activities reverses many aspects of the transformed phenotype that results from the loss of PTEN (Nakamura *et al*., 2000; Aoki *et al*., 2001; Neshat *et al*., 2001; Podsypanina *et al*., 2001; Stiles *et al*., 2002). Finally, the requirement for continued PI3K signaling elicited by PTEN loss in cancer cell lines is in keeping with the genetic connections established between PI3K, AKT and PTEN in *D. melanogaster* and *C. elegans*.

Thus, loss of PTEN leads to a continued dependence of PTEN-null cells on PI3K pathway activation. This continued dependence provides a notable therapeutic opportunity.

Somatic mutation of PTEN or PI3K pathway genes in human prostate cancer

Inactivating mutations in PTEN

The discovery of somatic alterations in the PI3K pathway in prostate cancer began with observations of LOH in the region of 10q. This event occurs in CaP with high frequency (30–60%) (Gray *et al*., 1995; Komiya *et al*., 1996) and two distinct commonly LOH regions have been identified at 10q22–q24 and 10q25, respectively, implying the presence of putative tumor suppressor genes at these loci (Komiya *et al*., 1996).

As mentioned above, *PTEN* maps to the 10q23.3 locus and is likely the tumor suppressor gene targeted by this genetic event (Li and Sun, 1997; Li *et al*., 1997; Steck *et al*., 1997). Somatic alterations of *PTEN* are common in other primary tumors including gliomas (Liu *et al*., 1997; Rasheed *et al*., 1997; Wang *et al*., 1997), endometrial cancers (Risinger *et al*., 1997; Tashiro *et al*., 1997), thyroid carcinoma (Dahia *et al*., 1997) and melanoma (Guldberg *et al*., 1997; Tsao *et al*., 1998).

Interest in genetic alterations in PTEN in prostate cancer began with the observation that PC-3 and

LNCaP cell lines (2 of the 3 commonly used prostate cell lines) harbor either a deletion (PC-3) or a point mutation in PTEN (LNCaP) (Li *et al*., 1997; Steck *et al*., 1997). Somatic *PTEN* alterations have been reported for both localized and metastatic prostate cancers. These genetic alterations include homozygous deletions, LOH, and inactivating missense and nonsense mutations. Point mutations in primary tumors were found in one of 40 primary tumors (Dong *et al*., 1998) and in five of 37 primary tumors (Gray *et al*., 1998), while homozygous deletions but not mutations were seen in eight of 60 tumors (Wang *et al*., 1998). Finally, Cairns *et al* reported LOH in 23 of 80 primary tumors with either deletion or mutation of the remaining allele in 10 of the 23 LOH + tumors. Thus, it is reasonable to conclude that a substantial minority ($\sim$15%) of primary tumors harbors PTEN mutations. This is of notable interest, as only a minority of primary tumors are associated with progression to lethal prostate cancer.

Somatic *PTEN* alterations appear more common in metastatic cancers. Suzuki *et al*. (1998) noted that 12 of 19 patients with metastatic disease had a mutation in PTEN in at least one metastatic site. Xenografts derived from metastatic foci have a high rate of PTEN loss and, specifically, homozygous deletion (Vlietstra *et al*., 1998). In keeping with these data, our group has assessed copy number alterations and LOH patterns in primary, hormone-sensitive lymph node metastatic prostate cancer and hormone-refractory metastatic prostate cancer, using high-density (100 K) single-nucleotide polymorphism arrays (Beroukhim R *et al*., unpublished data). As shown in Figure 1, biallelic loss is first seen in lymph node metastases and occurs in 50% of metastatic hormone-refractory prostate cancer. The analysis of larger numbers of primary tumors is required before we can determine whether a fraction of these tumors harbors deletions as well. Together, the bulk of the data suggests that the prevalence of *PTEN* mutation increases in the metastatic disease setting. Similarly, when studied by immunohistochemistry, loss of PTEN protein occurs in approximately 20% of localized prostate tumors. In this setting, PTEN loss is highly correlated with advanced stage and high Gleason grade (McMenamin *et al*., 1999). Thus, there may be a subfraction of primary tumors that lose PTEN and are hence destined to become metatstatic and hormone-independent.

Alterations in Akt in human prostate cancer

While amplification of the AKT1 or AKT2 genes has been noted in pancreatic ductal carcinomas (Cheng *et al*., 1996; Miwa *et al*., 1996; Ruggeri *et al*., 1998) and in ovarian and gastric cancer specimens (Staal, 1987; Cheng *et al*., 1992; Thompson *et al*., 1996), amplification of these loci has not been observed in prostate cancer. To date, activating point mutations have not been described in either AKT1, 2 or 3 in prostate cancer.

The activation of Akt has been studied in prostate cancer specimens using immunohistochemical means to detect phosphorylated Akt (pS473). In some studies, staining was detected in nearly all PIN and invasive prostate cancer samples (Sun *et al*., 2001; Van de Sande *et al*., 2005), while in another study the intensity of pS473 staining was positively correlated, with high preoperative serum levels of PSA (Liao *et al*., 2003) or was significantly greater, with higher Gleason grades 8–10 than PIN (Malik *et al*., 2002; Kreisberg *et al*., 2004). The relationship between IHC detection of Akt activation and PTEN mutation has not been established in prostate cancer.

Alterations in PI3K in human prostate cancer

Amplification (Shayesteh *et al*., 1999) and mutation (Samuels *et al*., 2004) of the gene encoding the catalytic subunit of the type 1 PI3K alpha subunit (PIK3CA) have been described as frequent somatic events in ovarian cancer, in breast cancer, hepatocellular carcinoma and glioblastoma among many cancer types. (Bachman *et al*., 2004; Broderick *et al*., 2004; Campbell *et al*., 2004). In prostate cancer, however, neither amplification nor mutation has been reported to date.

Alterations in IGFI in human prostate cancer

An association between plasma levels of IGF-1 and the risk of death from prostate cancer has been observed in prospective, population-based cohort studies (Chan *et al*., 1998; Wolk *et al*., 1998). Here, those men who are in the top quintile of IGF1 levels have a statistically

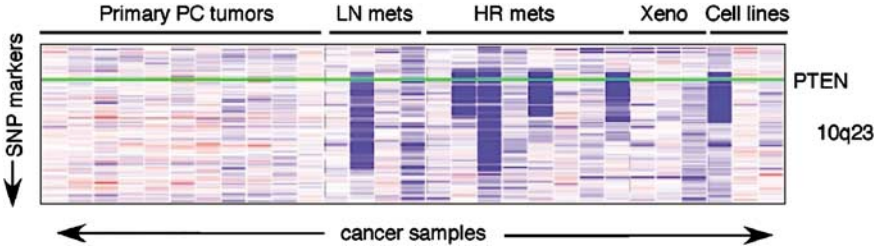

Figure 1 High-density single-nucleotide polymorphism (SNP) array analysis reveals homozygous deletions of the PTEN gene in prostate cancer samples. Each column represent one tumor or cell line sample (as indicated), while each row represents a single SNP marker. Only markers shown in the 10q23 interval are depicted. Increasing shades of blue represents gene copy-loss while increasing shades of red represent gene copy-gain. The genomic position of the PTEN gene is indicated by the green line. Abbreviations: PC – prostate cancer, LN – lymph node, xeno – xenografts, HR – hormone refractory. Genomic DNA from each sample was isolated, and processed for SNP array hybridization as previously described (Garraway *et al*., 2005)

significant increase in the risk of death from prostate cancer. More recent studies obtained in the so-called post-PSA era of prostate cancer diagnosis have failed to find these same associations. Thus, there remains a lack of clarity surrounding this finding.

Murine models of prostate cancer based on PI3K pathway activation

PTEN knockout mice

Conventional deletion of both alleles of *Pten* leads to developmental defects and death at embryonic days 6.5–9.5 days (Di Cristofano *et al*., 1998; Podsypanina *et al*., 1999). *Pten* heterozygous (+/−) mice develop prostatic intraepithelial neoplasia with nearly 100% penetrance, but these lesions apparently do not progress to macroinvasive cancers (Di Cristofano *et al*., 1998; Podsypanina *et al*., 1999). The viability of the *Pten*+/− mice is compromised as a result of lymphoproliferation and tumors of intestines, mammary, thyroid, endometrial and adrenal glands. Thus, it has been difficult to look at the resulting prostate phenotype in older aged mice.

Heterozygosity of *Pten* also cooperates with a number of engineered secondary events to enhance the phenotype. Heterozygous or homozygous loss of *Cdkn2b* ($p27^{Kip1}$), *Nkx3.1* and *Ink4a*/$p19^{arf}$ all exacerbate the PTEN prostatic phenotype. For example, *Pten*+/− mice, in the background of the *Cdkn2b*−/− genotype, develop prostate cancer within 3 months with 100% penetrance (Di Cristofano *et al*., 2001). PIN was observed in *Pten*+/−, *Ink4a*/$p19^{arf}$+/− or −/− earlier than in *Pten*+/− mice alone; however, progression to invasive cancer was not observed (You *et al*., 2002). PIN was observed earlier in *Pten*+/−,*Nkx3.1*+/− than in *Pten*+/− mice alone and invasive cancers with lymph node metastases are found in *Pten*+/−,*Nkx3.1*+/−, *Cdkn2b*+/− mice (Abate-Shen *et al*., 2003; Gao *et al*., 2004). Finally, despite the convergence of PTEN and TSC2 on a common downstream signaling pathway (mTOR) reduction of *Tsc2* cooperates to induce invasive prostate cancers in *Pten*+/− mice (Ma *et al*., 2005a).

LoxP-PTEN knockout mice

To determine the consequence of prostate-specific deletion of *Pten*, mice harboring floxed alleles of *Pten* (Lesche *et al*., 2002) have been generated and intercrossed with mice bearing a transgene directing the constitutive prostate-specific expression of Cre-recombinase (*ARR2PB*-Cre). In mice lacking both alleles of *Pten,* PIN develops with earlier onset than in *Pten*+/− mice and leads to invasive prostate cancer and ultimately to metastatic cancer (Trotman *et al*., 2003; Wang *et al*., 2003). Similarly, homozygous deletion of *Pten* achieved using a PSA promoter-driven Cre-recombinase leads to invasive prostate cancer with a 100% penetrance (Ma *et al*., 2005b) and similar pictures of progression were seen in mice bearing *MMTV*-Cre and *Pten* flox alleles (Backman *et al*., 2004).

These data strongly support the role of PTEN as a tumor suppressor, with particular relevance to prostate cancer initiation and progression.

Prostate-specific Akt transgenic mice

A transgenic line expressing a myristoylated and hence constitutively activated form of human Akt-1 was generated, in which the rat probasin promoter was used to restrict expression to the prostate (Majumder *et al*., 2003). In this model, activated AKT1 is spatially overexpressed in the ventral and lateral prostates, starting as early as postnatal day 2. The overexpression and activation of downstream molecules results in the development of dysplastic lesions with severe atypia, histopathological features consistent with PIN. AKT activation also led to changes in gene expression that are also known to occur in human prostate cancers. Notable among the upregulated transcripts was prostate stem cell antigen (PSCA), a gene that is expressed in prostate ductal tips during prostate development (Reiter *et al*., 1998). In human prostate cancers, PSCA is expressed in almost all cases of high-grade PIN and is overexpressed in approximately 40% of local and as many as 100% of bone metastatic prostate cancers (Gu *et al*., 2000).

The PIN phenotype does not progress to cancer, but 30–40% of older Akt-transgenic mice develop a protuberant abdomen as a result of a bladder outlet obstruction. In contrast to the results obtained with loss of function alleles of *Pten*, Tg-Akt1 did not develop invasive or metastatic prostate cancer. This important difference in phenotype may reflect the biologic differences between activating only Akt1 as opposed to inactivating PTEN, with the subsequent activation of PI3K and Akt. Alternatively, strain differences and hence germline genetic modifiers could, at least in part, account for some of these differences in the Akt1 transgenic mice maintained in the FVB background.

Genetic suppression of the PTEN phenotype in murine systems

The development of therapeutics for reversing treating of PTEN null tumors has been an area of intensive investigation. This results primarily from the array of drugable kinases that are suitable targets downstream of PTEN loss. To date, limited experiments have been carried out in mice to try and ascertain whether genetic deletion of any given pathway component (e.g. Akt or PI3K) is sufficient to suppress the PTEN phenotype. First, studies of teratoma formation suggest that Akt-1 is a major effector of the proliferation and tumor phenotype in *PTEN* homozygous (−/−) ES cells (Stiles *et al*., 2002). It has been more difficult to address the necessity of PI3K downstream of PTEN loss as mice lacking catalytic subunits of p110 are not viable; however, it has been possible to examine the requirement for the regulatory subunits of PI3K. Specifically, loss of the p85α and p85β subunits of PI3K in *Pten*+/−

mice does not alter the PIN formation, but a fraction of the proliferating cells in PIN is reduced in *Pten*+/− *p85β*−/− mice (Luo *et al*., 2005a).

Akt, a regulator of mTOR and its role in prostate cancer

The mTOR pathway is an important component of the downstream signaling cascade that is dysregulated by loss of function mutations in PTEN. These connections between activated Akt and activation of mTOR, likely involve Akt-dependent inactivation of TSC2 or proceed through the Akt or PDK1-dependent activation of $p70^{S6K}$. In either case, cells lacking PTEN or harboring activated alleles of Akt have high levels of mTOR activity and a resulting dysregulation of cell size, organ size and cell growth controls.

The mammalian target of rapamycin (mTOR) was identified after the discovery of its yeast homologs TOR1 and TOR2 (Brown *et al*., 1994; Chiu *et al*., 1994; Sabatini *et al*., 1994). mTOR is a member of the atypical protein kinase family and phosphorylates substrates critical for protein synthesis, including ribosomal subunit S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (Schmelzle and Hall, 2000). Thus, the output of mTOR signaling is likely mediated through regulation of protein translation. How mTOR activation might lead to cellular transformation is still not completely clear. Downstream of mTOR broad alterations in protein translation might account for oncogenesis. Alternatively, specific critical proteins that are regulated through translation might be deregulated and may contribute to the required oncogenic signals. For example, it has also been proposed that dysregulation of the protein synthesis machinery through mTOR may generate cell-cycle progression signals that contribute to cancer (Ruggero and Pandolfi, 2003). Another potential target of mTOR is PP2A, which is downregulated by small T-antigen and is important for the transformation of human cells with SV40 T-antigens and Ras (Hahn *et al*., 2002). mTOR phosphorylates PP2A *in vitro* likely leading to downregulation of PP2A preventing the dephosphorylation of 4E-BP1 (Peterson *et al*., 1999). Thus, it is possible that PP2A is a critical target of mTOR-mediated signals.

In this vein, emerging data suggest that Hif1α stabilization may play an important role downstream in the induction of the neoplastic phenotype *in vivo*, and that mTOR inhibition, at least in part, reverts aspects of transformation through regulation of Hif1α levels (Hudson *et al*., 2002; Brugarolas *et al*., 2004; Majumder *et al*., 2004). Hif1α transcriptional targets are constitutively activated in the Akt-dependent prostatic intraepithelial neoplasia model, and this deregulation is completely mTOR dependent. Among the targets of Hif1α transcription are included nearly all the members of the glycolysis pathway from hexokinase to lactate dehydrogenase. An important implication is that the regulation of glycolysis and glucose uptake may likewise play an important role in the growth of such tumors.

With respect to Hif1α regulation, both Akt-dependent/mTOR-independent pathway and Akt dependent/mTOR-dependent pathways have been described (reviewed in Semenza, 2003; Abraham, 2004). Recent data suggest that hypoxia-induced activation of Hif1α requires mTOR activity (Zhong *et al*., 2000; Hudson *et al*., 2002), that insulin activates Hif1α through the Akt/mTOR-dependent pathway (Treins *et al*., 2002) and that in the setting of loss of TSC2, Hif1α protein and mRNA levels are elevated, leading to upregulated expression of Hif1α target genes (Brugarolas *et al*., 2003). Elevated Hif1α activity is, in this setting, reversed by mTOR inhibition (Brugarolas *et al*., 2003), but the mechanism leading to mTOR-dependent elevated Hif1α activity remains unclear.

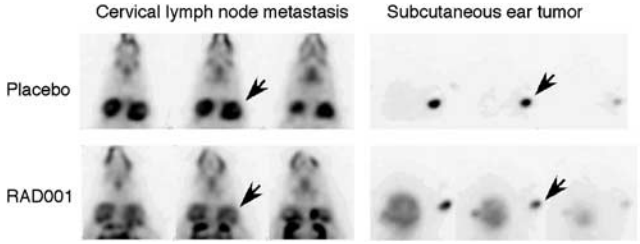

Figure 2 ^{18}FDG-PET uptake is blocked by two doses of the rapamycin derivative RAD001 in primary and metastatic tumors. Murine cancer cells were subcutaneously implanted in the mouse ear and allowed to grow in the implantation sites and metastasize to cervical lymph nodes. Tumor bearing mice were treated with either placebo or RAD001 at a dose of 10 mg/kg body weight for 2 days and glucose uptake was determined by ^{18}FDG-PET analysis. Arrows indicate the position of the cervical lymph node metastasis (left panel) and subcutaneous ear tumor (right panel). This figure was generously provided by Paul McSheehy, Novartis Institute of Biomedical Research, Oncology, CH-4002, Basel, Switzerland

Glucose uptake and in particular hexokinase activity can be 'sensed' or imaged *in vivo* using the radiotracer ^{18}Fluorodeoxyglucose and positron emission tomography (^{18}FDG-PET). Generally, ^{18}FDG-PET uptake by tumors is thought to simply reflect upregulated metabolic activity; however, the regulation by mTOR suggests that genetic alteration of the pathway may specifically turn on glycolytic enzymes. Indeed, preclinical proof-of-concept experiments have shown that ^{18}FDG-PET uptake can be blocked by two doses of the rapamycin derivative RAD001 (Figure 2) (McSheehy *et al*., 2005). This provides the opportunity to use ^{18}FDG-PET as an *in vivo* pharmacodynamic marker for mTOR inhibitors.

Strategies for blocking the PI3K/Akt/mTOR pathway in prostate cancer

Introduction

The frequent occurrence of inactivating mutations in the PTEN tumor suppressor in hormone-refractory prostate cancer has provided one of the few genetically defined in-roads to cancer therapeutics in prostate cancer. The PI3K pathway presents a number of attractive kinase targets for small molecule development that will be discussed below. Furthest along in clinical development

are the inhibitors of mTOR, all of which are derivatives of rapamycin.

IGF1R inhibition

IGF1R has long been known as a critical survival and proliferation signal transducer. While genetic alterations in cancer have not yet been observed, murine fibroblasts that lack IGFIR are resistant to the transforming activities of a number of oncogenes including SV40 large-T antigen (Coppola *et al*., 1994). These latter data suggest that IGFIR is required for transformation and thus might be a suitable drug target. The IGFI/IGFIR axis is clearly important in human prostate development and in the development of prostate cancer. Indeed, constitutive secretion of IGFI itself in transgenic animals is sufficient to induce prostate cancer (DiGiovanni *et al*., 2000). Examination of IGFI−/− mice has also revealed that IGFI is required for the normal development of the murine prostate (Ruan *et al*., 1999).

IGFIR small-molecule inhibitors are in development and have reported activity against myeloma, small cell lung cancer and certain sarcomas; however, activity against prostate cancer has not been described to date (Garcia-Echeverria *et al*., 2004; Mitsiades *et al*., 2004; Scotlandi *et al*., 2005; Warshamana-Greene *et al*., 2005). It is also not clear whether such inhibitors would have preferential activity against PTEN-null cells or conversely whether loss of PTEN might render cells resistant to upstream IGFIR inhibition. Similarly, humanized selective antibodies directed against IGFIR are also in development, but whether such antibodies will have therapeutic efficacy remains to be seen (Burtrum *et al*., 2003; Maloney *et al*., 2003; Cohen *et al*., 2005).

PI3K inhibition

There are ongoing efforts to develop kinase inhibitors against the catalytic subunits of PI3K. Early leads in this area include the commonly used laboratory tool compounds wortmannin and LY294002, which target the p110 catalytic unit of PI3K. These molecules have relatively broad specificity and short *in vivo* half-life and are poorly suited for clinical development. Second generation PI3K inhibitors, though not widely published appear to have much improved isoform specificity and also improved pharmacologic properties and activity in xenograft models (Ward *et al*., 2003; Workman, 2004). Again, however, whether there will be enhanced sensitivity in cells lacking PTEN or whether there is activity in prostate cancer models remains to be seen. These agents have not yet reached the clinic.

An alternative strategy for interrupting PI3K signaling is to block recruitment of the PI3K holoenzyme to receptor tyrosine kinases by disrupting the phosphotyrosine binding of the SH2-domain of the p85 subunit of PI3K. Here, peptidomimetics have been successfully used to block this association (Eaton *et al*., 1998), but will likely require significant optimization prior to *in vivo* administration.

AKT inhibition

Inhibition of Akt remains an attractive therapeutic approach to interdiction of the PI3K pathway. Despite serious efforts directed at building ATP-competitive kinase inhibitors, no selective small molecules have made it to the clinic. High potency inhibitors have been described (Thimmaiah *et al*., 2005; Luo *et al*., 2005b) and appear to have antitumor activity in xenografts models, although with some evidence of metabolic toxicities. In addition, a set of allosteric inhibitors that requires the PH domain of Akt for activity have been described (Barnett *et al*., 2005; Lindsley *et al*., 2005) and *in vitro*, induce apoptosis in the PTEN-null LNCaP prostate cell line. Finally, a number of 'akt' or pathway inhibitors have been described including calmodulin inhibitors (Kau *et al*., 2003), myo-inositol derivatives (Meuillet *et al*., 2004; Tabellini *et al*., 2004) and phosphatidylinositol ether analogs (Gills and Dennis, 2004) that inhibit the activation of Akt as measured by inhibition of Akt phosphorylation itself (as opposed to direct activation of Akt kinase activity). These latter agents might act either on Akt recruitment or on known or novel upstream activators of Akt. To date, there is no significant clinical experience with these agents in prostate cancer patients.

HSP90 inhibition

It appears that either events that lead to the activation of protein kinases, or kinases bearing activating mutations become particularly dependent on chaperone function for appropriate folding and activity. Notable in this regard is that phosphorylated and hence activated

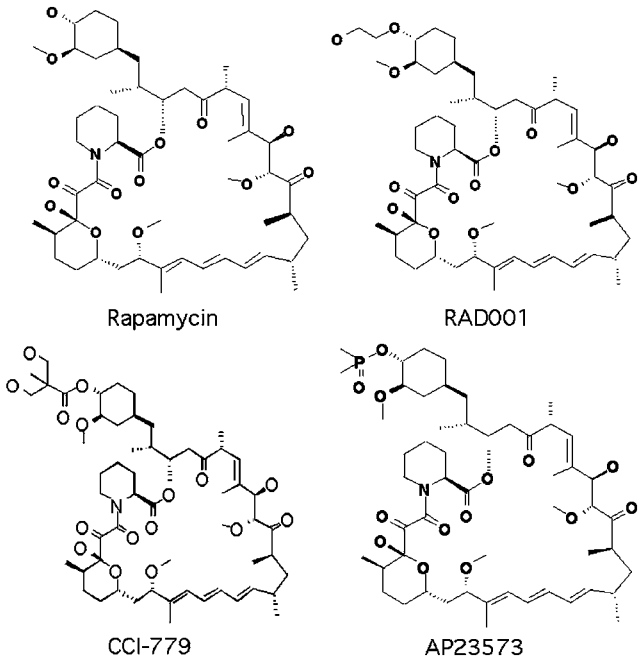

Figure 3 Structures of rapamycin and the rapamycin-derivatives in clinical development

Table 1 Current clinical status of PI3K/Akt/mTOR pathway inhibitors in cancer

Drug	*Manufacturer*	*Disease*	*Trial status*
CCI-779	Wyeth-Ayerst Laboratories	Malignant gliomas	Phase I/II
		Metastatic breast cancer	Phase III
		Androgen-independent prostate cancer	Phase II
RAD001	Novartis Pharmaceutical	Advance non-small-cell lung cancer	Phase I/II
		Endometrial cancer	Phase II
		Recurrent glioblastoma	Phase I/II
AP 23573	Ariad Pharmaceuticals	Progressive and recurrent gliomas	Phase I

Akt likewise appears to require the function of the HSP90 chaperone for continued activity (Sato *et al*., 2000; Hostein *et al*., 2001; Basso *et al*., 2002; Solit *et al*., 2003; Gills and Dennis, 2004). Geldanamycin or its derivative 17-AAG are ansamycins that selectively inhibit HSP90 function by occupying the nucleotide binding site. The ansamycins appear to have anticancer activity and have prompted the development of new series of HSP90 inhibitors with improved pharmacologic properties and facile syntheses (reviewed in Chiosis *et al*., 2003; Cheung *et al*., 2005).

Recently, phase I data were reported for 17-AAG. Here, the dose-limiting toxicities were diarrhea and hepatoxicity. There were two patients with melanoma who had evidence for disease stabilization and no patients with prostate cancer were treated in this study (Banerji *et al*., 2005).

mTOR inhibition

Rapamycin, first isolated from *Streptomyces hygroscopicus,* binds to FKBP12 (also known as FK506-binding protein) and induces binding to and inactivation of mTOR. Cell lines harboring inactivating mutations in PTEN are particularly sensitive to rapamycin or the derivative CCI-779 (Neshat *et al*., 2001). Similarly, chicken fibroblasts transformed by activating alleles of AKT or PI3K also have increased sensitivity to these agents (Aoki *et al*., 2001). *In vivo*, treating *Pten* + /− null mice with CCI-779 reduces the number of intestinal lesions (Podsypanina *et al*., 2001) and the treatment of Akt transgenic animals with RAD001 completely reverts the PIN phenotype to normal (Majumder *et al*., 2004). These experiments have encouraged the clinical development of mTOR inhibitors in the context of PTEN or PI3K pathway alterations. However, the mTOR-dependent regulation of Hif1α also raises the possibility that rapamycin or its derivatives might have a role as anti-angiogenic agents.

Current status of mTOR inhibitors in prostate cancer

Three derivatives of rapamycin CCI-779, RAD001 and AP 23573 (see Figure 3) either have completed or are completing phase I trials (Table 1). A phase II trial of CCI-779 was initiated in prostate cancer using intravenous weekly dosing. Toxicity may have resulted in the premature closure of this trial and no data have been reported in the published literature. A number of new phase II trials are underway, including a combination trial of Gefitinib and RAD001 in prostate cancer, as well as a Taxotere and RAD001 trial. Both of these trials will use ^{18}FDG-PET imaging as a pharmacodynamic end point. It will likely be some time before we know whether mTOR inhibition will have therapeutic value in selected patients or in combination with second agents.

Summary

The genetic evidence strongly supports the role of PTEN mutation and hence AKT activation in metastatic or high-grade prostate cancers. Therapeutics for these aggressive forms of prostate cancer are severely lacking and agents targeting this pathway are likely to find a role in the management of prostate cancer. The major hurdles remain the discovery, optimization and clinical development of small molecule inhibitors for the major kinases dysregulated by PTEN loss, namely AKT and PI3K.

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